RJP/TMH/DJZ:kam 01/17/03 163881
PATENT

Attorney Reference Number 245-55928
Application Number 09/673,763

#11
JM
1/28/03

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Rockey and Bannantine

Art Unit: 1645

Application No. 09/673,763

Filed: October 16, 2000

For: CHLAMYDIA PROTEINS AND THEIR USES

Examiner: Rodney P. Swartz, Ph.D.

Date: January 17, 2003

CERTIFICATE OF MAILING

I hereby certify that this paper and the documents referred to as being attached or enclosed herewith are being deposited with the United States Postal Service on Jan 21, 2003 as First Class Mail in an envelope addressed to: COMMISSIONER FOR PATENTS, WASHINGTON, D.C. 20231.

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WASHINGTON, D.C. 20231

DECLARATION OF DANIEL D. ROCKEY, Ph.D. UNDER 37 C.F.R. § 1.132

I, DANIEL D. ROCKEY, Ph.D. declare as follows:

1. I am an inventor of and have read and understand U.S. Patent Application No. 09/673,763 entitled CHLAMYDIA PROTEINS AND THEIR USES, including the Response to Restriction Requirement and Voluntary Amendment filed on May 29, 2002.

2. A copy of my *curriculum vitae* is attached hereto as **Exhibit A**. I have been an Associate Professor in Microbiology at Oregon State University from July 2002 to present. I previously was an Assistant Professor in the same department from June 1997 through June 2002, which included the time during which the above-referenced patent application was filed.

3. I understand that Claims 5-12 and 19-30 are currently pending in the application, and that Claims 5-12 and 19-30 are rejected under 35 U.S.C. § 112, first paragraph, as allegedly containing subject matter that was not described in the specification in such a way as to reasonably convey that the inventors possessed this invention at the time the application was filed.

4. As a researcher well versed in the study of microbiology and infectious diseases, I believe the teachings provided in the specification of U.S. Patent Application No. 09/673,763 indicate that Dr. Bannantine and I possessed the claimed subject matter at the time the application was filed. Specifically, the teachings of the patent application show that Dr. Bannantine and I knew how to make the claimed immune stimulating compositions and perform the claimed methods to generate immune responses to *Chlamydia psittaci* and *Chlamydia trachomatis* IncA proteins. This knowledge was described in the specification at the time the application was filed. For example, the discussions in the specification on page 23, line 17 through page 24, line 25, and page 19, line 29 through page 21, line 12 provide guidance enabling one of ordinary skill in the art to prepare and administer compositions to induce an immune response using the IncA *Chlamydia* protein sequences disclosed in the application, such as SEQ ID NOs: 8 or 14.

5. Further evidence supporting my belief is a publication, Bannantine *et al. Infect. Immun.* 66: 6017-6021, 1998, attached as **Exhibit B**. This publication discusses experiments in which my colleagues and I used compositions prepared as described in the application (*e.g.*, at page 19, line 29 through page 21, line 12) and methods disclosed in the specification (*e.g.*, at page 23, line 17 through page 24, line 25) to induce an immune response in New Zealand White rabbits[1]. Specifically, the rabbits were injected with a preparation including purified *C. trachomatis* IncA protein as an antigen, as taught in the specification at page 23, line 17 through page 24, line 25. An immune response was elicited to the injected composition, as evidenced by the generation of antibodies to the IncA antigen. These antibodies were purified and later used to screen samples from humans and primates previously infected with *Chlamydia*. Figure 3 of **Exhibit B** is an immunoblot analysis, demonstrating that sera from both monkeys and humans showed a strong, specific signal when probed with the IncA antibody, indicating that IncA is present in monkeys and humans infected with *Chlamydia*.

[1] Drs. Walter E. Stamm and Robert J. Suchland participated in the experiments, but are not inventors of the claimed subject matter.

6. It is widely accepted in the field of study of infectious diseases that humans generate antibodies (*i.e.*, an immune response) in a similar manner to other mammals such as mice and rabbits. Furthermore, it is accepted that antibodies generated in rabbits by injection of antigen proteins that induce an immune response in humans may be used to detect the presence of those proteins in samples obtained from infected humans. Therefore, I believe that purified *C. psittaci* and *C. trachomatis* IncA protein, if administered to humans using the claimed compositions and methods, would generate a similar immune response to that shown in rabbits in **Exhibit B**. Hence, the compositions and methods described in the specification indicate that Dr. Bannantine and I possessed the claimed subject matter at the time the application was filed.

7. All statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true. Further, these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that any such willful false statements made may jeopardize the validity of the application or any patent issuing thereon.

Date: 1/18/03, 2003

Daniel D. Rockey, Ph.D.

EXHIBIT A

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University of Washington, Seattle, WA	B. S., Fisheries	1980
University of Wyoming, Laramie, WY	M. S., Microbiology	1983
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Pr fessional experience

Teaching Assistant/ Research Assistant, University of Wyoming	1981-1983
Research Assistant, Zymogenetics Corp., Seattle, WA.	1983-1985
Teaching Assistant/ Research Assistant, Oregon State University	1986-1989
Research Associate, Laboratory of Dr. Stephen Kaattari, Oregon State University	1989-1990
Postdoctoral Fellow, Rocky Mountain Laboratories, National Institutes of Health, Hamilton, MT	1991-1995
Senior Staff Fellow, Rocky Mountain Laboratories, National Institutes of Health, Hamilton, MT	1995-1997
Assistant Professor of Microbiology Oregon State University, Corvallis, OR.	June 1997- July 2002
Associate Professor of Microbiology Oregon State University, Corvallis, OR.	July 2002- present

Publications

Brown, W. J Y. A. W. Skeiky, P. Probst, and **D. D. Rockey.** 2002. Chlamydial Antigens Colocalize within IncA-laden Fibers extending from the Inclusion Membrane into the Host Cytosol. In press, Infection and Immunity.

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Recent meetings/ abstracts:

1. Brown, W. J. and **D. D. Rockey**. 2001. Chamydial Antigens Are Localized to the Host Cell *Cytosol* by Trafficking Through IncA-Laden Fibers. Abstract # D 201, Annual Meeting of the American Society for Microbiology, Orlando, FL May 2001.

2. Werth, E. P., J. Lenart, D. Alzhanov, **D. D. Rockey**, and D. E. Hruby. 2001. Altered Host Cell Morphology Following Transfection of Cells with Chlamydial IncC. Abstract # D 201, Annual Meeting of the American Society for Microbiology, Orlando, FL May 2001.

3. Lenart, J., A. A. Andersen, and **D. D. Rockey**. 2000. Aberrant growth of Tetracycline-resistant *Chlamydia trachomatis* isolates Grown in Near Limiting Concentrations of Tetracycline. Abstract # D 62, Annual Meeting of the American Society for Microbiology, Los Angeles CA. May 2000.

4. Lenart, J., A. A. Andersen, and **D. D. Rock y**. 2000. Aberrant growth of tetracycline-resistant *Chlamydia trachomatis* isolates grown in near limiting concentrations of tetracycline. Annual Meeting of the American Society for Microbiology, Los Angeles. May 2000.

5. Brown, W. J., and **D. D. Rock y**. 2000. Characterization of a genus common antigen that localizes to the apparent septum in dividing chlamydiae. Annual Meeting of the American Society for Microbiology, Los Angeles. May 2000.

6. Viratyosin, W., R. J. Suchland, W. E. Stamm, and **D. D. Rockey**. 2000. Diverse mutations in *incA* amplified from clinical *Chlamydia trachomatis* isolates that occupy nonfusogenic inclusion. Annual Meeting of the American Society for Microbiology, Los Angeles. May 2000.

7. J. P. Bannantine[1] R. S. Griffiths, W. Viratyosin, W. Brown and **D. D. Rockey**. 1999 A Secondary Structure Motif Predictive of Protein Localization to the Chlamydial Inclusion Membrane Meeting of the American Society for Cell Biology, Dec. 1999. Washington D.C.

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INFECTION AND IMMUNITY, Dec. 1998, p. 6017–6021
0019-9567/98/$04.00+0

Vol. 66, No. 12

Chlamydia trachomatis IncA Is Localized to the Inclusion Membrane and Is Recognized by Antisera from Infected Humans and Primates†

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Received 22 June 1998/Returned for modification 29 July 1998/Accepted 11 September 1998

***Chlamydia psittaci* produces a collection of proteins, termed IncA, IncB, and IncC, that are localized to the chlamydial inclusion membrane. In this report we demonstrate that IncA is also produced by *Chlamydia trachomatis*. *C. trachomatis* IncA is structurally similar to *C. psittaci* IncA and is also localized to the inclusion membrane. Immunoblot analysis demonstrated that sera from *C. trachomatis*-infected patients and from experimentally infected monkeys both recognized *C. trachomatis* IncA.**

Chlamydiae depend heavily on their host cells for energy and essential nutrients, including amino acids and nucleoside triphosphates. Unlike species of the bacterial parasites *Shigella*, *Listeria*, and *Rickettsia*, which have direct access to the nutrient-rich environment of the host cytoplasm (8, 20, 21), chlamydiae are sequestered in a membrane-bound vacuole, termed an inclusion. Living within a vacuole presents some unique challenges not faced by organisms in the cytoplasm. One of these challenges includes the acquisition of nutrients from the host cell. Heinzen and Hackstadt (6) showed that the inclusion membrane is not passively permeable to molecules as small as 520 Da by microinjection studies of fluorescent tracer molecules. Therefore, nutrient acquisition is likely mediated through transport mechanisms at the inclusion membrane.

Another key to chlamydial pathogenesis and survival is their ability to avoid fusion with lysosomal compartments in order to persist and replicate within the host cell. Several experiments have shown the mature chlamydial inclusion to be nonfusogenic with markers from the endosomal-lysosomal pathway. Electron microscopic analysis showed that ferritin-labeled lysosomes do not fuse with the inclusion (23). Neither fluid-phase markers nor markers of the early or late endosomes are associated with the chlamydial inclusion (7, 15, 19). However, chlamydiae do sequester and modify host cell lipids and apparently reside in an exocytic arm of the host vesicular trafficking network (4, 5, 22). Modification of the vesicle to intersect an exocytic pathway requires chlamydial protein synthesis, which suggests that the chlamydiae synthesize proteins that determine the vesicular interactions of the inclusion (16).

It is thought that both acquisition of nutrients and avoidance of lysosomal fusion may be mediated by chlamydial proteins secreted into the inclusion membrane. This led to the identification and characterization of IncA, a *Chlamydia psittaci* protein that is present uniquely in infected cells, is localized to the inclusion membrane (12), is exposed to the host cell cytoplasm, and is phosphorylated by the host cell (13). Two additional inclusion membrane proteins, termed IncB and IncC, were recently identified in *C. psittaci* (1).

Despite considerable effort, *incA*, *incB*, and *incC* were never detected in *Chlamydia trachomatis* by conventional laboratory methods. The failure of these approaches led to the concern that *C. psittaci* IncA, IncB, and IncC might not directly model inclusion development in the human pathogenic species of the chlamydiae. With the completion of the *C. trachomatis* genome project (17), *incA* has been identified in this species. This report describes our characterization of IncA from *C. trachomatis*.

Organisms. *C. trachomatis* LGV-434, serovar L2, and *C. trachomatis* serovar D were cultivated in HeLa 229 cells as previously described (3). The trachoma biovar strains (serovars A, B, Ba, and C), the genital strains (serovars D, D-, E, F, G, H, I, Ia, J, and K), and the LGV biovar strains (serovars L1, L2, L2a, and L3) were also cultivated in HeLa cells. Specific strains studied included A/G-17/OT, B/TW-5/OT, Ba/Ap-2/OT, C/TW-3/OT, D/UW-3/Cx, Da/TW-448/Cx, D-/MT 157/Cx, E/UW-5/Cx, F/UW-6/Cx, G/UW-57/Cx, H/UW-4/Cx, I/UW-12/Ur, Ia/UW-202/NP, I-/MT 518/Cx, J/UW-36/Cx, K/UW-31/Cx, L1/440/Bu, L2/434/Bu, L2a/UW-396/Bu, L3/404/Bu, and *C. psittaci* GPIC.

Antiserum production. A maltose-binding protein (MBP)-IncA fusion protein was produced by using the pMAL-c2 vector system from New England Biolabs as described previously (1). *C. trachomatis* serovar D *incA* was amplified with 5′-AG CCATAGGATCTGGTTTCAGCGA-3′ and 5′-GCGCGGAT CCTAGGAGCTTTTTGTAGAGGGTGA-3′ and then cloned into pMAL-c2.

MBP-IncA was used as antigen for the production of monospecific antibody in New Zealand White rabbits (12). Antiserum against *C. trachomatis* serovar L2 was produced in cynomolgus monkeys (*Macaca fascicularis*). Monkeys were anesthetized and infected urethrally with *C. trachomatis* elementary bodies (EBs) three times over the course of 6 months. Symptoms of infection were monitored over time. Antisera from infected monkeys were tested for reactivity to chlamydiae by enzyme-linked immunosorbent assay (reference 18 and unpublished data) and immunoblotting. Human sera that demonstrated high titers of antibody to *C. trachomatis* or *Chlamydia pneumoniae* by microimmunofluorescence assay were selected from stored serum specimens at the University of Washington. Negative control antisera were taken from pa-

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† Technical paper 11411 of the Oregon State University Extension and Experiment Station.

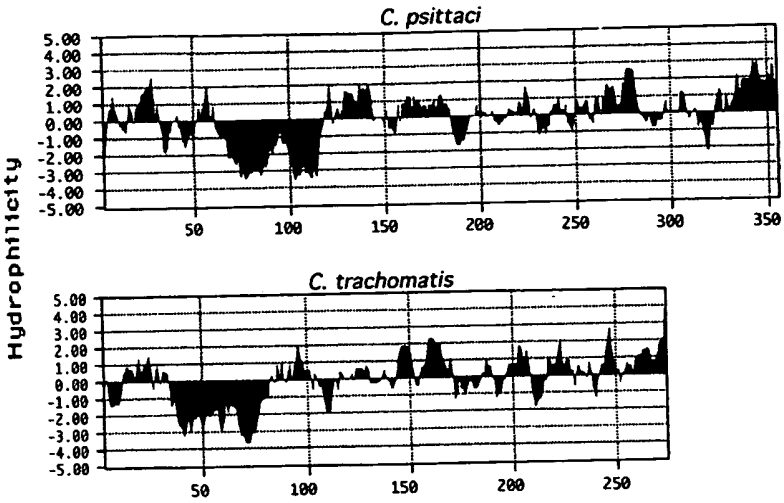

FIG. 1. Comparison of IncA proteins from *C. psittaci* and *C. trachomatis* by hydropathy plot analysis. A hydropathy profile of each protein shows a unique bilobed hydrophobic domain in the N-terminal half. Profiles were determined by the algorithm developed by Kyte and Doolittle (9), with a window size of seven amino acids. The vertical axis displays relative hydrophilicity, with negative scores indicating relative hydrophobicity.

tients who had no detectable reactivity by microimmunofluorescence against any of the *C. trachomatis* serovars listed above or *C. pneumoniae* TWAR. Antilipopolysaccharide monoclonal antibody was produced as described previously (2).

Immunoblotting and immunofluorescence microscopy. Polyacrylamide gel electrophoresis and immunoblotting were performed as previously described (11, 12). Chlamydiae grown in HeLa cells on sterile glass coverslips were methanol fixed 30 h postinfection and stained as previously described (12). Immunostained coverslips were visualized with the 63× objective of a Zeiss microscope equipped with an epifluorescence condenser and an MC 63 C photomicrographic camera.

Sequence analysis of *C. trachomatis incA*. All sequence analysis was conducted by using methods described by Bannantine et al. (1). *C. trachomatis incA* was identified by limited homology in the *C. trachomatis* genome sequence database (17). A BLAST search of the amino acid sequence showed *C. psittaci* IncA to be the strongest match in the database, but that match was weak, with an E value of only 2×10^{-5}. The 30-kDa size of IncA from *C. trachomatis* is smaller than that of *C. psittaci* IncA, and their identity and similarity were only 21 and 41%, respectively. Weak homology at the nucleotide sequence level explained why *C. trachomatis incA* was not detected by Southern hybridization or PCR amplification with probes and prim-

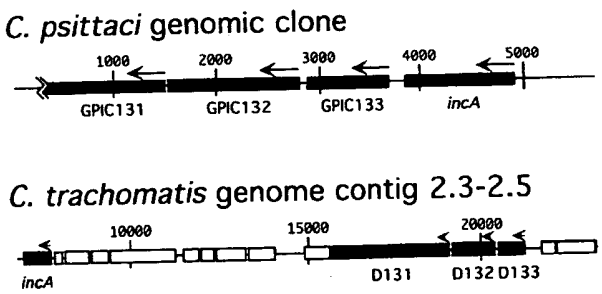

FIG. 2. ORF map of the chromosomal region surrounding *incA* in *C. psittaci* and *C. trachomatis*. ORFs 131, 132, 133, and *incA* are labeled. Note the scale difference between the maps. ORF 133 is immediately downstream of *incA* in *C. psittaci*, whereas it is upstream and separated by at least 10 kb in *C. trachomatis*. Base pairs are indicated above each map, and arrows indicate the direction of transcription. The ORF designation is preserved from the *C. trachomatis* serovar D genome database designations. Pustell protein matrix analysis was used to confirm that GPIC131 and GPIC132 correspond to D131 and D132, respectively.

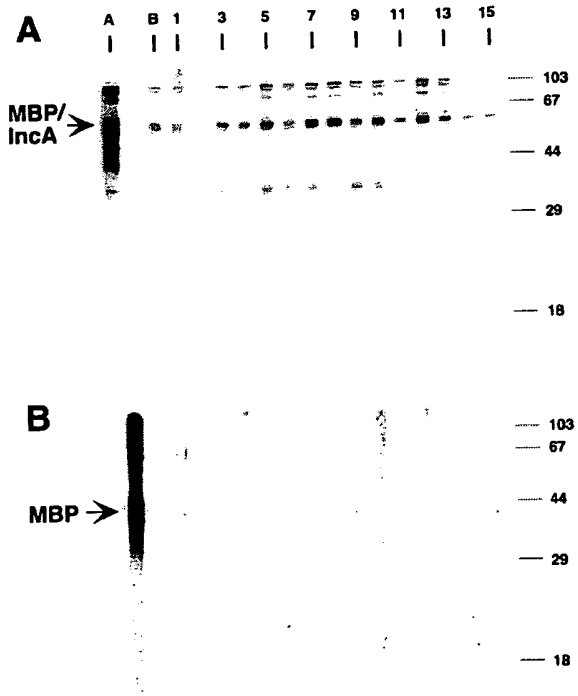

FIG. 3. Preparative immunoblot analysis of a purified MBP-*C. trachomatis* IncA fusion protein (A) and purified MBP (B), each probed with antisera from chlamydia-infected patients and monkeys. Lane A, anti-MBP; lane B, monkey convalescent-phase sera; lanes 1 and 2, sera from *C. pneumoniae*-infected patients; lanes 3 to 13, sera from *C. trachomatis*-infected patients; lanes 14 and 15, negative control sera.

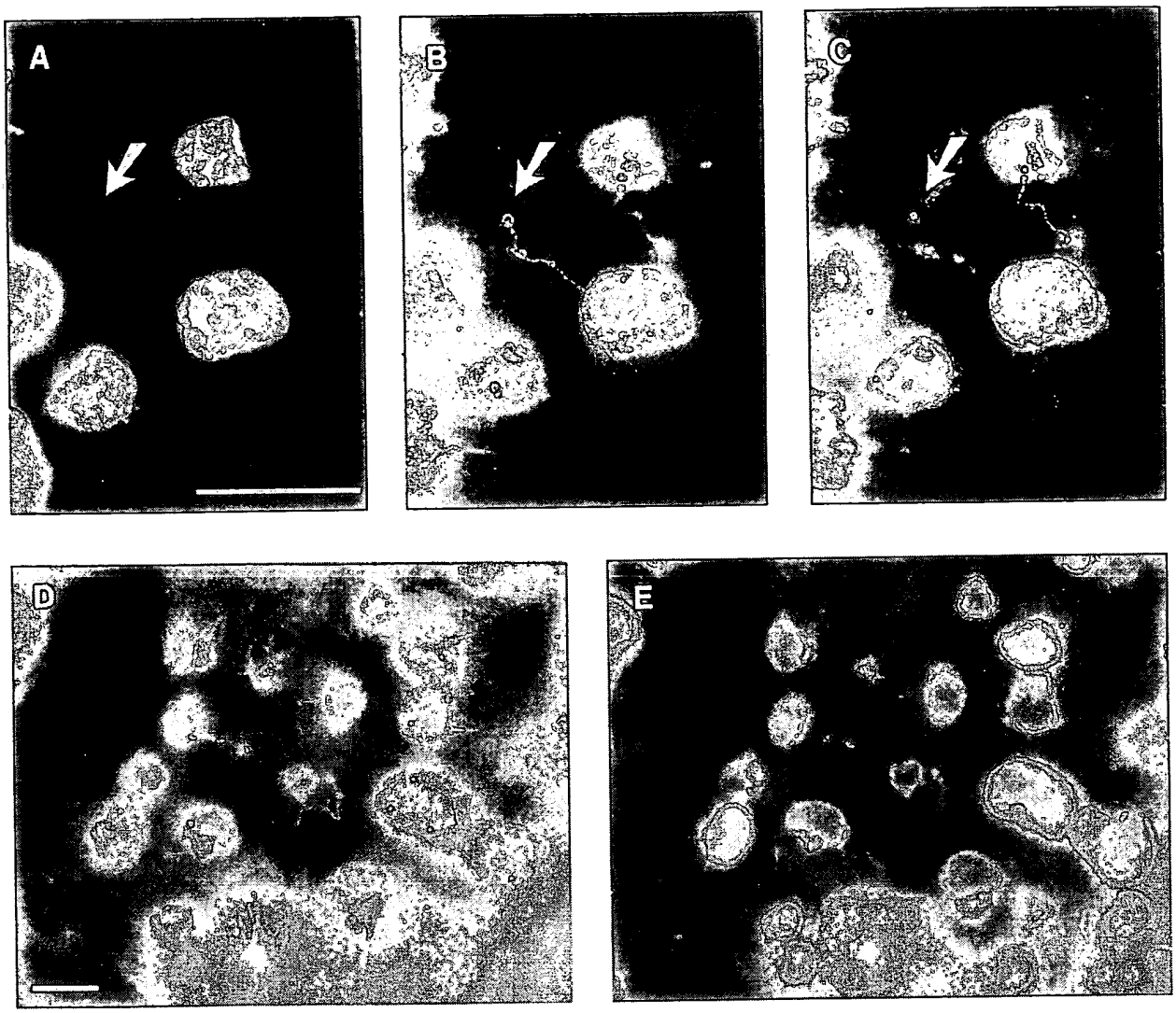

FIG. 4. Immunofluorescence microscopy with anti-IncA demonstrating that IncA is localized to the inclusion membrane in *C. trachomatis*-infected cells. Serovar L2-infected HeLa cells were fixed in methanol 25 h postinfection and stained with anti-major outer membrane protein (A) and/or anti-MBP-IncA (B to E). Panels A to C represent a single image, with panel C photographed in a different focal plane. Note the fibers extending between the two inclusions in different cells as well as from one infected cell to an apparently uninfected cell (uninfected cell at tip of arrow). Note also the antigenic fibers extending from several inclusions in one focal plane (D) and IncA in inclusions at different stages of maturation in another focal plane (E). Bars in panels A and D represent 10 μm for panels A to C and panels D and E, respectively.

ers from the *C. psittaci* genomic sequence. Although IncA sequence identity between *C. trachomatis* and *C. psittaci* is low, comparison of their hydropathy plots shows similar large hydrophobic regions near the N-terminal ends (Fig. 1). Such a long hydrophobic region, with its unique bilobed shape, may be useful in predicting other chlamydial proteins in the inclusion membrane since it is also present in IncB and IncC (1). The location of the hydrophobic domain is near the C-terminal end in IncB and IncC. To show that this hydrophobic domain is not fortuitous, several open reading frames (ORFs) identified in the *C. trachomatis* genome project have been screened by hydropathy plot analysis, and only tested ORFs that encode proteins with similar secondary structure are localized to the inclusion membrane (13a). Primers were designed from the serovar D *incA* sequence, and they amplified *incA* from serovar L2 as well as D. The sequence from these two serovars is highly conserved: only 5 of 273 amino acids are different. The same primers did not amplify a product with *C. pneumoniae* genomic DNA as a template.

The region surrounding *incA* is not conserved between *C. trachomatis* and *C. psittaci*. In previous work, we and others have isolated four independent *C. psittaci* genomic clones that

TABLE 1. Chlamydia strains used for reactivity with anti-*C. trachomatis* IncA

Biovar, strain, or cell type	Serovars	Immunofluorescence staining
Trachoma	A, B, Ba, C	+
Oculogenital	D, Da, D-, E, F, G, H, I, Ia, I-, J, K	+
LGV	L1, L2, L2a, L3	+
C. psittaci GPIC		−
Uninfected HeLa cells		−

collectively define a group of four physically linked genes as shown in Fig. 2 (12, 13a). The completion of the *C. trachomatis* genome sequence has allowed a comparison of the arrangement of these genes in *C. psittaci* and *C. trachomatis*. Each of the four ORFs is present in the *C. trachomatis* genome, but the physical linkage has been disrupted. In *C. psittaci*, *incA* is immediately upstream of an ORF designated GPIC133 (Fig. 2), with an intergenic region of 157 bp (see orf2 in reference 12). ORF 133 is present in both *C. psittaci* and *C. trachomatis* and is relatively conserved, with 58% identity between the deduced amino acid sequences. The *incA* coding sequence in *C. trachomatis* is downstream and separated from ORF 133 (D133) by 12,678 bp, with *incA* located at contig 2.3 in the genome and D133 located at contig 2.5. Note the scale difference between the two genomic segments in Fig. 2.

Immunoblot analysis of infected cells and purified *C. trachomatis* EBs was performed with rabbit anti-MBP-IncA as a probe. A 27-kDa band was present only in the infected cells and not in lysates of EBs or uninfected cells (data not shown).

In order to determine if IncA was recognized by sera from convalescent animals and humans, purified MBP-IncA fusion protein was loaded onto a preparative sodium dodecyl sulfate-polyacrylamide gel and used to examine reactivity with sera from patients and monkeys infected with *C. trachomatis*. The majority of the sera from chlamydia-infected patients (10 of 11) and all monkey convalescent-phase sera recognized the IncA protein (Fig. 3A) but not the MBP portion of the fusion (Fig. 3B). IncA was faintly recognized by sera from one of the *C. pneumoniae*-infected patients (Fig. 3A, lane 1).

Antisera against IncA and a monoclonal antibody against chlamydial lipopolysaccharide were used to immunostain methanol-fixed layers of *C. trachomatis*-infected HeLa cells. Anti-IncA reacted with the membrane of the inclusion but not the chlamydial developmental forms (Fig. 4A to C). Antigenic fibers extending away from the inclusion, which are similar in structure to those found in *C. psittaci*-infected cells (12), were also present in *C. trachomatis*-infected cells (Fig. 4B to D). Their function and origins remain unknown. Also evident in Fig. 4B and C are antigenic fibers that traverse between otherwise apparently separate cells. It is likely that these are daughter cells in which inclusions can either divide with the dividing cell (10) or stay in one daughter cell and leave the other uninfected. *C. psittaci* IncA can also be found in fibers that extend between pairs of infected cells (data not shown). One major difference between these two processes is that in *C. psittaci* (strain GPIC), each daughter cell usually remains infected. In *C. trachomatis*, however, uninfected progeny cells are common. Because IncA is also found in fibers that extend to the uninfected daughter cells (Fig. 4B and C), the result is a cell lacking chlamydial developmental forms but containing chlamydial antigen.

In addition to the LGV biovar strain (serovar L2) shown in Fig. 4, several other *C. trachomatis* serovars of clinical interest were analyzed by immunofluorescence microscopy for staining with anti-MBP-IncA (Table 1). Anti-MBP-IncA labeled the inclusion membranes of all serovars tested.

The inclusion membrane mediates all contact between the host cell and chlamydiae; therefore, the acquisition of nutrients and the nonfusogenic nature of the chlamydial inclusion may be elucidated by studying chlamydial proteins that reside in the inclusion membrane. Because the routing of transport vesicles throughout the cell is mediated by proteins present on the transport vesicle membrane (14), IncA as well as IncB and IncC are excellent candidate proteins for mediating inclusion trafficking within infected cells. We undertook these studies to define the presence and intracellular location of IncA in all of the major *C. trachomatis* serovars and to assess whether an antibody response to IncA was present in infected patients and primates. We speculate that *C. pneumoniae* also produces Inc-like proteins and are initiating an investigation into this system. Finally, we continue to pursue questions surrounding the role of the Inc proteins in the chlamydial infection process as well as their role as possible protective antigens in the host response to chlamydial infection.

Nucleotide sequence accession number. The nucleotide sequence of *C. trachomatis* LGV-434, serotype L2, *incA* has been deposited in the GenBank database under accession no. AF067958.

We gratefully acknowledge Richard Stephens and Claudia Fenner at the University of California, Berkeley, for their efforts in the completion of the *C. trachomatis* genome project. We thank Linda Cles of the University of Washington for providing the human serum samples. We also thank Harlan Caldwell and Michael Parnell for assistance with the production of primate convalescent-phase sera.

A portion of this work was supported by Public Health Service (PHS) grants AI-31448 and N01-AI-75329 to W.E.S. and PHS grant AI42869-01 to D.D.R.

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Editor: P. E. Orndorff